The Protein-Synthesizing Activity of Ribosomes Isolated from the Mammary Gland of Lactating and Pregnant Guinea Pigs

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1. Polyribosome preparations were made from the deoxycholate-treated post-nuclear fractions obtained by the disruption of mammary glands from lactating and pregnant guinea pigs. 2. A high proportion of large polyribosomes was obtained from the glands of lactating animals whereas mainly small polyribosomes were obtained from the glands of pregnant animals. The isolated preparations incorporated [^{14}C]phenylalanine into protein. The polyribosomes from the glands of pregnant animals were less active than those from the glands of lactating animals but the activity of the former was stimulated more by poly(U) than was the latter. 3. The ribosomes from mammary gland could be dissociated into subunits after incubation, under conditions necessary for protein synthesis, in the presence of puromycin. The subunits could be recombined to give a preparation that actively polymerized [^{14}C]phenylalanine in the presence of poly(U). The subunits from guinea-pig mammary gland could be combined with subunits from liver of either guinea pig or rat. Hybrid ribosomes were also formed from subunits derived from glands of pregnant and lactating animals. The hybrids were as active as were the ribosomes formed by reassociation of subunits from the same tissue, suggesting that in this respect the ribosomes from pregnant animals were not defective. 4. Polyribosomes from mammary glands of lactating animals when incubated with cell sap from the same source were tested for their ability to synthesize α -lactalbumin. The polyribosomes were incubated in the presence of [^3H]leucine and α -lactalbumin was isolated from the supernatant. The protein was finally treated with cyanogen bromide and the C-terminal and N-terminal fragments were separated and their radioactivity was determined. Both fragments were radioactive consistent with the synthesis of α -lactalbumin. 5. The results are discussed in relation to protein synthesis in the mammary gland after parturition.

The events in the mammary gland that lead up to the initiation of the synthesis of milk protein at about the time of parturition provide the biochemist with a most intriguing challenge. It was with the hope of increasing our understanding of this system that Brew & Campbell (1967b) studied the synthesis of a specific whey protein, α -lactalbumin, by subcellular fractions prepared from the mammary glands of lactating guinea pigs. The present paper is an extension of this work to a study of the nature and activity of the ribosomes involved in the synthesis of whey protein and to a comparison of these ribosomes with those isolated from the mammary glands of pregnant guinea pigs.

Brew & Campbell (1967b) showed, in agreement with earlier work (Fraser & Gutfreund, 1958), that the most active subcellular fraction for protein synthesis was that normally termed 'mitochondrial'.

Polyribosomes have therefore been isolated from this fraction. Their activity for the incorporation of ^{14}C -labelled amino acids has been studied. In an attempt to locate any differences in the properties of the ribosomal subunits from glands of pregnant and lactating animals the ribosomes from both sources were dissociated by the method of Lawford (1969). Hybrid ribosomes were then formed and a study was made of their ability to synthesize polyphenylalanine in the presence of poly(U). The synthesis of α -lactalbumin by isolated polyribosomes from the mammary glands of lactating guinea pigs has also been studied. Some aspects of this work have been published in a preliminary form by Fairhurst & Campbell (1967). While this work was in progress Baird & Herriman (1968) described some of the characteristics for protein synthesis of a subcellular fraction from pregnant and lactating

rabbits, and Herrington & Hawtrey (1969, 1971) have studied the activity of the microsomal fraction from bovine mammary gland.

MATERIALS AND METHODS

Materials

Chemicals. Chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. and were of AnalaR grade wherever possible. The scintillators were purchased from Nuclear Enterprises (G.B.) Ltd., Sighthill, Edinburgh, U.K. Puromycin dihydrochloride was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; poly(U) was from Miles Laboratories Inc., Kankakee, Ill. 60901, U.S.A.; Sephadex was from Pharmacia Fine Chemicals, Uppsala, Sweden; CM-cellulose grade CM52 and DEAE-cellulose grade DE32 were from W. and R. Balston (Modified Cellulose) Ltd., Springfield Mill, Maidstone, Kent, U.K.; and polyacrylamide gel grade Bio-Gel P10 was from Bio-Rad Laboratories, Richmond, Calif. 94804, U.S.A. Other chemicals were as specified by Campbell, Lowe & Serck-Hanssen (1967).

Radiochemicals. The following were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.: L-[U- 14 C]leucine (sp. radioactivity 312 mCi/mmol), L-[U- 14 C]phenylalanine (sp. radioactivity 475 mCi/mmol) and L-[4,5- 3 H]leucine (sp. radioactivity 22 Ci/mmol).

Animals. The rats used in these studies were male, Wistar strain, and were purchased from Tuck and Sons Ltd., Rayleigh, Essex, U.K. The rats weighed between 150 and 200 g and were starved overnight before being killed. Guinea pigs were obtained from Fisons Pharmaceuticals Ltd., Loughborough, Leics., U.K. The pregnant animals were estimated to be within 3 days of parturition. The lactating animals were used about 4 days after parturition and were in full lactation.

Subcellular fractionation

(a) *Preparation of liver polyribosomes from rats and guinea pigs.* A slight modification of the procedure of Wettstein, Staehelin & Noll (1963) was used. The tissue was minced and homogenized as described by Ragnotti, Lawford & Campbell (1969). The 12000 g-supernatant was adjusted to contain 1.3% deoxycholate and 5 ml samples were layered over 3.0 ml of 0.5 M-sucrose in medium A (50 mM-tris-HCl buffer, pH 7.8 at 25°C, 25 mM-KCl, 5 mM-MgSO₄), and 4.0 ml of 2.0 M-sucrose in medium A. After centrifugation at 165000 g_{av.} for 3.5 h, the supernatant was removed by suction, the polyribosome pellet drained and the wall of the tube wiped clean with tissue.

(b) *Preparation of cell sap from rat and guinea-pig liver and mammary gland.* The tissue was minced and homogenized in 2.5 vol. of 0.15 M-sucrose in medium B (50 mM-tris-HCl buffer, pH 7.8 at 25°C, 25 mM-KCl, 10 mM-MgSO₄). The 120000 g-min supernatant was centrifuged at 105000 g_{av.} for 60 min and the resulting supernatant was siphoned off to provide the cell sap.

In some cases cell sap was passed through a column (15 cm × 2.0 cm) of Sephadex G-25 equilibrated with

medium B at 2°C and eluted with medium B. The coloured cell sap was collected.

(c) *Preparation of polyribosomes from guinea-pig mammary gland.* After removal of fat by dissection the tissue was minced as for liver and homogenized in 2.5 vol. of 0.25 M-sucrose in medium A. The suspension was centrifuged at 800 g for 10 min. The supernatant was decanted through a pad of glass wool to remove fat globules and used for the preparation of polyribosomes as described for the 120000 g supernatant from liver in (a) above.

Amino acid incorporation by subcellular fractions

Samples of the appropriate fractions were suspended in medium A (0.4 ml) and incubated in air at 37°C for 60 min with ATP (2 mM), GTP (if the cell sap had been treated by gel filtration) (0.25 mM), phosphoenolpyruvate (10 mM), poly(U) (100 µg where appropriate), MgSO₄ [5 mM in the absence of poly(U) and 14 mM in the presence of poly(U)], cell sap (0.2 ml) and 14 C-labelled amino acid (0.125 µCi) in a total volume of 1 ml. At the end of the incubation the tubes were placed in ice.

Dissociation of polyribosomes into ribosomal subunits

The method with minor modifications was that of Lawford (1969) as described by von der Decken, Ashby, McIlreavy & Campbell (1970). This involves the incubation of polyribosomes under conditions for protein synthesis using untreated cell sap in the absence of 14 C-labelled amino acids but in the presence of 0.7 mM-puromycin. The ribosome concentration was 1.6 mg/ml as measured on the basis of $E_{260}^{1\%}$ 134 (light-path 1 cm). Incubation was carried out in a volume of 10 ml in a 100 ml conical flask for 60 min. The flask was placed in an ice bath and the Mg²⁺ concentration was decreased to 2 mM and the K⁺ concentration increased to 100 mM by the addition of 1.5 vol. of a mixture of 50 mM-tris-HCl buffer, pH 7.8 at 25°C, and 0.15 M-KCl. To separate the ribosomal subunits 4 ml of the diluted suspension was loaded on to each of six tubes containing 33 ml of a sucrose density gradient [15–35% (w/v) sucrose in 50 mM-tris-HCl buffer, pH 7.8 at 25°C, 0.15 M-KCl, 1 mM-MgSO₄] in the SW 27 rotor of the Beckman L2/65B ultracentrifuge. Centrifugation was for 8 h at 95100 g_{av.}. The E_{260} was measured in a Gilford model 2000 recording spectrophotometer by pumping the gradients from the bottom through a flow cell at a constant flow rate of about 1 ml/min. The two subunit peaks were collected and pooled in chilled tubes and 0.5 vol. of medium A was added to each. The two subunits were pelleted by centrifugation for 3 h at 165000 g_{av.}. Pellets were stored for up to 48 h in the deep freeze.

Reassociation of ribosomal subunits

The subunit pellets were gently resuspended with a glass rod, in medium A, to give a concentration of 0.75 mg of ribosomes/ml for the small subunit and 1.72 for the large subunit. The subunit suspensions (0.1 ml) were either incubated separately or together, under the conditions described above for protein synthesis.

Purification of α -lactalbumin from guinea-pig milk

The procedure was modified from the method described by Brew & Campbell (1967a). Milk (70 ml) was diluted to 120 ml with water and the pH adjusted to 4.6 with *m*-acetic acid. The precipitate of casein was removed by centrifugation and the supernatant adjusted to pH 7.0 with 1.0 *M*-NaOH, dialysed against water overnight and freeze-dried. [In all dialysis procedures acetylated Visking tubing was used (Vanaman, Wakil & Hill, 1968).] The dry protein was taken up in 10 ml of 50 *mM*-NH₄HCO₃ and loaded on a column (4.4 cm $\times$ 140 cm) of Sephadex G-100 that had been previously equilibrated with the same buffer. The column was eluted with 50 *mM*-NH₄HCO₃ and the E_{280} of the effluent monitored. The fractions containing α -lactalbumin were pooled and freeze-dried. The protein was 98% homogeneous on polyacrylamide-gel disc electrophoresis (Brew & Campbell, 1967a).

Large-scale incubation of polyribosomes from lactating mammary gland for the biosynthesis of α -lactalbumin in vitro

Polyribosomes were prepared from the mammary glands of three lactating guinea pigs. Two incubation mixtures were set up. (a) In a total volume of 30 ml the mixture contained polyribosomes (75 mg) suspended in medium A (12 ml), cell sap that had been treated with Sephadex G-25 (6 ml) and 0.5 μ Ci of [4,5-³H]₂leucine. The final concentration of the other substances in the incubation medium was: ATP, 2 *mM*; phosphoenolpyruvate, 10 *mM*; GTP, 0.25 *mM*; MgSO₄, 5 *mM*; mercaptoethanol, 1 *mM*. (b) This was identical with mixture (a) but ATP was replaced by water. Incubation was carried out for 1 h at 37°C in 250 ml conical flasks with shaking in air. The incubation was stopped by placing the flasks in ice and the ribosomes were separated by centrifugation for 2 h at 165 000 *g*_{av} in the Ti50 rotor of the Beckman L2/65B centrifuge. The supernatants were decanted and stored in the frozen state.

Isolation of α -lactalbumin from the supernatant after incubation of polyribosomes

The supernatants (a) and (b) were thawed and 50 mg of the partially purified α -lactalbumin from milk was added as carrier. The pH was adjusted to 4.5 with *m*-acetic acid. The precipitate was removed by centrifugation and the supernatants were adjusted to pH 7.0 with 1.0 *M*-NaOH. Each was dialysed extensively against water and freeze-dried. The protein was dissolved in 2 ml of 50 *mM*-NH₄HCO₃ and loaded on columns (2.5 cm $\times$ 120 cm) of Sephadex G-100 equilibrated and eluted with the same buffer. Fractions (8 ml) were collected, the E_{280} was measured and the radioactivity determined on 0.2 ml samples after the addition of 5 ml of Bray's (1960) solution. The fractions containing α -lactalbumin were pooled, freeze-dried and dissolved in 2.5 ml of 15 *mM*-sodium acetate buffer, pH 5.0, dialysed against the same acetate buffer, loaded on a column (2.3 cm $\times$ 20 cm) of CM-cellulose equilibrated with 15 *mM*-sodium acetate buffer and eluted with a linear gradient of 0–50 *mM*-NaCl in the acetate buffer. Fractions (6 ml) were collected, the E_{280} was measured and the radioactivity determined on 0.2 ml samples. Supernatant (a) was further treated as follows.

The fractions from the CM-cellulose column containing α -lactalbumin were pooled, freeze-dried and then dissolved in 2 ml of 20 *mM*-tris-HCl buffer, pH 7.8 at 25°C, and dialysed against the same tris buffer. The solution was loaded on a column (1.6 cm $\times$ 15 cm) of DEAE-cellulose equilibrated with 20 *mM*-tris, pH 7.8, and eluted with a linear gradient of 0–0.4 *M*-NaCl in 20 *mM*-tris, pH 7.8. Fractions (6 ml) were collected, the E_{280} was measured and the radioactivity determined on 0.5 ml samples. The fractions containing α -lactalbumin were pooled, dialysed against water and freeze-dried. The protein was amino-ethylated and cleaved with CNBr as described by Brew & Hill (1970). After cleavage the solution was diluted fivefold with water and freeze-dried. The CNBr-treated freeze-dried protein was dissolved in 1 ml of 1% (v/v) formic acid and loaded on a Bio-Gel P10 column (1.5 cm $\times$ 130 cm) equilibrated and eluted with 1% formic acid. Fractions (3.5 ml) were collected, the E_{280} was measured and the radioactivity determined. In this case, samples (1 ml) were placed in scintillation vials and freeze-dried. Water (0.2 ml) was added to each vial followed by 5 ml of Bray's (1960) solution.

Determination of the radioactivity of protein

After incubation of the polyribosomes with ¹⁴C-labelled amino acid, protein was extracted and its radioactivity was determined in a Beckman model LS 200B scintillation spectrometer to a standard error of 5% or better as described by Ragnotti *et al.* (1969). For determination of [³H]leucine in fractions obtained during the purification of α -lactalbumin, Bray's (1960) solution was used. The radioactivity-counting efficiency was 28%.

RESULTS

Analysis of polyribosomes from the mammary glands of lactating and pregnant guinea pigs

Previous work by Brew & Campbell (1967b) indicated that the major protein-synthetic activity of subcellular fractions from lactating gland was present in a fraction corresponding to the post-nuclear fraction from rat liver. Preliminary results showed that this fraction was also richer in terms of polyribosomes than the post-mitochondrial fraction so that the low-speed (8000 *g*-min) supernatant was always used as the source of polyribosomes. Although some polyribosomes could be isolated from this 8000 *g*-min supernatant in the absence of deoxycholate in the preparation, indicating the presence of some membrane-free polyribosomes, the yield was much greater when deoxycholate was included. Fig. 1 shows a typical pattern of a sucrose density gradient of a polyribosome preparation from a gland of a lactating animal. When glands were taken from animals that had only just ceased lactation (1–2 days) few polyribosomes could be detected. A sucrose-density-gradient analysis of a preparation of polyribosomes from a gland of a pregnant animal is shown in Fig. 2. This demonstrates that (a) the proportion of polyribosomes that are larger than pentamers is much greater in the

Fig. 1. Sucrose-density-gradient centrifugation of a polyribosome preparation from the mammary gland of a lactating guinea pig. The post-8000g-min supernatant was treated with deoxycholate and the polyribosomes were pelleted as described in the Materials and Methods section. The gradient was 15–50% sucrose in medium A and centrifugation was from right to left for 4 h at 63 600g_{av.}. The volume of the gradient was 29 ml and 3.25 mg of polyribosomes in medium A (1 ml) was used.

Fig. 2. Sucrose-density-gradient centrifugation of a polyribosome preparation from the mammary gland of a pregnant guinea pig. Conditions for the preparation of the polyribosome fraction and its centrifugation were exactly as for Fig. 1; 1.7 mg of polyribosomes in medium A was used.

preparation from the glands of lactating than pregnant animals and (b) the ratio of total polyribosomes to monomers is much greater in the preparation from lactating animals. We also observed that the yield of total ribosomal material in the polyribosome preparation based on wet weight of tissue was 3 times as great in glands from lactating animals than from pregnant animals. The RNA/protein ratio of the ribosomes from both sources was the same, namely 1:1. Various procedures were used in an attempt to improve the yield of large polyribosomes in preparations from pregnant animals with the possibility in mind that the aggregates may have been degraded during the isolation procedure. Thus in one case the cell sap from liver was added to the homogenate to inhibit ribonuclease activity. Another procedure was to include EDTA (0.5 mM) in the homogenization medium as recommended by Matthaei & Schoech (1967) for placenta and for heart muscle by Earl & Morgan (1968). In one experiment heparin was also included in the homogenization medium as described by Turkington & Riddle (1970). In no case were we able to improve the proportion of large polyribosomes so that none of these procedures was used regularly.

Protein-synthesizing activity of polyribosome preparations from guinea-pig mammary gland

Preliminary experiments to determine the optimum conditions for the incorporation of ¹⁴C-labelled amino acids by isolated preparations of polyribo-

somes from mammary gland indicated that these were similar to those for preparations from liver. Cell sap prepared from the mammary glands of lactating animals was active in the incorporation system but that from pregnant animals was much less active. Since liver cell sap was also active with particles from mammary gland it was used in the incubations described in this section.

A comparison of the activity of preparations from lactating and pregnant animals for the incorporation of [¹⁴C]phenylalanine into protein is shown in Table 1. The preparations from pregnant animals were always much less active than those from lactating animals but were stimulated much more by the addition of poly(U).

Since, in the absence of added poly(U), the polyribosome preparations from pregnant animals were considerably less active than those from lactating animals the possibility was investigated that the ribosomes from pregnant animals were in some way defective. To test this, hybrids were formed between ribosomal subunits from different tissues. To check the procedure a study was made first with ribosomal hybrids from mammary gland of lactating guinea pig and rat liver, since it was with the latter tissue that the method was worked out. Fig 3 shows the separation of the ribosomal subunits from various tissues on a sucrose density gradient. The protein-synthesizing activity of the hybrids is shown in Table 2(a). In comparing the activity of the subunits alone one should note that 2.3 times as much of the large subunit was incubated as that of the small subunit. In each case there was a substantial synergistic effect on the activity when the subunits were incubated together. The activity for incorporation of phenylalanine was in all cases totally depen-

Table 1. *Comparison of protein-synthesizing activity of polyribosomes from the mammary glands of lactating and pregnant guinea pigs*

Conditions for incubation were as described in the Materials and Methods section. Each tube (1 ml) contained polyribosomes together with cell sap from liver and $0.125 \mu\text{Ci}$ of $[^{14}\text{C}]$ phenylalanine, and in appropriate cases $100 \mu\text{g}$ of poly (U). Results are expressed as c.p.m./ $100 \mu\text{g}$ of ribosomes. The quantity of ribosomes was based on $E_{260}^{1\%}$ 134 (light-path 1 cm). The radioactivity of the protein extracted at zero-time incubation has been subtracted in all cases.

Expt. no.	Lactating animals			Pregnant animals		
	No poly(U) (a)	+Poly(U) (b)	Ratio (b/a)	No poly(U) (a)	+Poly(U) (b)	Ratio (b/a)
1	700	2732	3.9	340	7130	21
2	2169	6440	3.0	1217	13567	11

Fig. 3. Separation of ribosomal subunits by sucrose-density-gradient centrifugation. The ribosomes after incubation with puromycin as described in the Materials and Methods section were subjected to centrifugation at $95100g_{av}$ for 8 h on a sucrose gradient (15–35%) in 50 mM-tris, 0.15M-KCl and 1 mM-MgSO₄. After centrifugation the E_{260} was measured in a recording spectrophotometer by pumping the gradients from the bottom through a flow cell at a constant flow rate of about 1 ml/min. The ribosomes were derived from (a) rat liver, (b) guinea pig liver, (c) mammary gland from lactating guinea pigs, (d) mammary gland from pregnant guinea pigs.

dent on the presence of poly(U). Although the activities of the hybrids were not as high as those of the recombined liver subunits, there is no doubt that the different subunits interacted to provide active ribosomes. Similar results were obtained when subunits from mammary gland of lactating animals were hybridized with those from guinea-pig liver (Table 2b). This encouraged us to test the effect of hybridizing ribosomes from the mammary

glands of pregnant and lactating animals. The reassociated subunits from either source had similar protein-synthetic activity to the hybrids (Table 2c).

Studies on the synthesis of α -lactalbumin

Polyribosomes from the mammary glands of lactating animals were incubated in the presence of cell sap from similar glands and $[^3\text{H}]$ leucine.

Table 2. *Protein-synthesizing activity of ribosomal subunits from mammary glands of lactating and pregnant guinea pigs and rat and guinea-pig liver*

The ribosomal subunits were prepared and incubated as described in the Materials and Methods section. The incubation was in the presence of [^{14}C]phenylalanine at a Mg^{2+} concentration of 14 mM. The ratio of the amount of large subunit to small subunit incubated was always 2.3:1 (i.e. 0.172 mg of large subunit and 0.075 mg of small subunit). The radioactivity of the protein obtained from the incubation mixture in the absence of ribosomes was subtracted from the results in (a) and (c), and in (b) the zero-time-incubation value was subtracted. The numbers in parentheses indicate the straight addition of the activity for the various combinations of subunits incubated independently. There was a complete dependence of activity on the presence of poly(U) (100 μg).

(a) Mammary gland of lactating guinea pig or rat liver

Source of ribosomes		Contents of incubation mixture							
Mammary gland of lactating guinea pig	Small subunit	+		+				+	
	Large subunit		+	+					+
Rat liver	Small subunit				+		+		+
	Large subunit					+	+	+	
Total radioactivity (c.p.m.)		254	1068	7524 (1322)	216	764	10052 (980)	8161 (1018)	7383 (1284)

(b) Mammary gland of lactating guinea pig or guinea-pig liver

Source of ribosomes		Contents of incubation mixture							
Mammary gland of lactating guinea pig	Small subunit	+		+				+	
	Large subunit		+	+					+
Guinea-pig liver	Small subunit				+		+		+
	Large subunit					+	+	+	
Total radioactivity (c.p.m.)		457	718	6741 (1175)	561	1081	9034 (1642)	10883 (1538)	7869 (1279)

(c) Mammary gland of lactating or pregnant guinea pig

Source of ribosomes		Contents of incubation mixture							
Mammary gland of lactating guinea pig	Small subunit	+		+				+	
	Large subunit		+	+					+
Mammary gland of pregnant guinea pig	Small subunit				+		+		+
	Large subunit					+	+	+	
Total radioactivity (c.p.m.)		132	237	1580 (369)	87	194	1583 (281)	1437 (326)	1553 (324)

Mercaptoethanol was included in the medium in case it was of importance for the formation of the correct disulphide linkages in α -lactalbumin, which contains four such bonds. To demonstrate the energy dependence of the incorporation of [^3H]leucine into protein a control incubation was carried out in the absence of ATP. This procedure is preferred to the omission of the energy-generating system, since the effective Mg^{2+} concentration is barely changed by the omission of ATP, whereas it is markedly altered by the omission of phosphoenolpyruvate. After incubation, the polyribosomes were separated by centrifugation, carrier α -lactalbumin was added to the supernatant and the proteins were fractionated. The results of the purification of α -lactalbumin from the incubation mixture that contained ATP are shown in Fig. 4. Fig. 4(a) shows the separation achieved by gel filtration and indicates that α -lactalbumin was intermediate in molecular size

among the substances containing [^3H]leucine. After fractionation on CM-cellulose (Fig. 4b) and DEAE-cellulose (Fig. 4c) the α -lactalbumin was almost pure. At this stage advantage was taken of the fact that guinea-pig α -lactalbumin contains only one methionine residue at position 90 (K. Brew, personal communication) and so may be cleaved into two fragments as described for bovine α -lactalbumin by Brew & Hill (1970). The separation of the C-terminal and N-terminal fragments from the intact protein by gel filtration in formic acid is shown in Fig. 4(d). There is a perfect coincidence between the E_{260} and radioactivity peaks for the C-terminal fragment and less certainly for the N-terminal fragment and undegraded α -lactalbumin. In the purification of the protein from the incubation mixture which did not contain ATP, gel filtration showed that at least five components contained [^3H]leucine but after fractionation on CM-cellulose the fractions con-

Fig. 4. Purification of α -lactalbumin from the soluble medium after incubation of polyribosomes from mammary glands of lactating guinea pigs in the presence of ATP. $\circ$, E_{280} ; $\bullet$, radioactivity (c.p.m.). In all cases fractions (indicated by the bar) were pooled for further purification. (a) Elution from a column of Sephadex G-100. Samples (0.2 ml) of each fraction (8.8 ml) were taken for the determination of radioactivity. (b) Elution from a column of CM-cellulose; samples (0.2 ml) of each fraction (6.0 ml) were taken for determination of radioactivity. The arrow indicates the point at which 0.5M-NaCl in 15 mM-sodium acetate buffer, pH 5.0, was applied as eluent. (c) Elution from a column of DEAE-cellulose. Samples (0.5 ml) of each fraction (5.8 ml) were taken for determination of radioactivity. (d) Fractionation of cyanogen bromide fragments of α -lactalbumin on a column of Bio-Gel P10. The peak at fraction 47 is the C-terminal fragment, that at fraction 38 the N-terminal fragment and that at fraction 33 undegraded α -lactalbumin. Samples (1.0 ml) of each fraction (3.5 ml) were taken for the determination of radioactivity.

taining α -lactalbumin had an insignificant radioactivity. The purification was therefore abandoned at this point.

The recovery of label throughout the purification of α -lactalbumin from the two incubation mixtures is recorded in Table 3. At the point where the purification of α -lactalbumin from the incubation mixture containing no ATP was abandoned, the specific radioactivity was comparatively very low. With the incubation in the presence of ATP, the α -lactalbumin at a specific radioactivity of 557 c.p.m./mg was treated with cyanogen bromide. The radioactivity associated with each fragment was determined by measuring the areas under the respective curves. Since the primary sequence of guinea-pig α -lactalbumin is known (K. Brew,

personal communication) it can be deduced that the C-terminal fragment (residues 91–123) contains four leucine residues and the N-terminal fragment (residues 1–90) contains ten leucine residues. Thus reasonably assuming equivalent recoveries of the two peptides the radioactivity contributed per residue of leucine in each fragment could be deduced. This showed that per mol of leucine the radioactivity of the C-terminal fragment was nine times that of the N-terminal fragment.

DISCUSSION

No difficulty has been experienced in the preparation of polyribosomes from the mammary glands of lactating guinea pigs (Fig. 1). Preparations from

Table 3. *Recovery of radioactivity during the purification of α -lactalbumin from the supernatant after incubation of polyribosomes from lactating mammary gland*

The total radioactivity represents that present in the fractions from the columns containing α -lactalbumin and as indicated in Fig. 4 by the bars. The specific radioactivity is based on the total radioactivity in c.p.m./mg of protein.

Stage of purification	Radioactivity			
	With ATP		Without ATP	
	Total (c.p.m.)	Sp. radioactivity (c.p.m./mg of protein)	Total (c.p.m.)	Sp. radioactivity (c.p.m./mg of protein)
Supernatant derived from incubation				
After Sephadex G-100	1.66×10^6	4.54×10^4	3.7×10^4	1.1×10^3
After CM-cellulose	5.8×10^4	30×10^3	1320	70
After DEAE-cellulose	3.9×10^3	557	—	—

pregnant animals contained much less ribosomal material and, in contrast to the preparations from lactating animals, contained a much smaller proportion of large polyribosomes (Fig. 2). We are aware that this may represent a technical failure on our part, in that the polyribosomes existing in the gland may be broken down to ribosomes during isolation. Alternatively it may be that large polyribosomes are not abundant in the glands of the pregnant animals. Examination of sections of glands from pregnant animals by electron microscopy has not so far provided unequivocal evidence on this point. In an attempt to preserve polyribosomes during cell disruption various procedures that had been found helpful for the isolation of polyribosomes from several different kinds of tissues were applied. These included the addition, at all stages of the isolation procedure, of cell sap from liver, which is known to inhibit the action of ribonuclease, the addition of small quantities of EDTA, as recommended by Matthaei & Schoech (1967) and Earl & Morgan (1968), and of heparin, as recommended by Turkington & Riddle (1970). The last authors have succeeded in obtaining some large polyribosomes from the mammary glands of pregnant mice. They attribute their success to a precise set of conditions used during the isolation procedure and to the inclusion of heparin at all stages. Although we followed their methods carefully we could not obtain a higher proportion of large polyribosomes from the mammary glands of pregnant guinea pigs.

The yield of ribosomal RNA based on wet-tissue weight was much lower in the glands from pregnant than lactating animals. This may not reflect a difference in the total ribosomal population of the two types of gland for we used a procedure which was selective for polyribosomes and most of the monomeric ribosomes would not have been recovered. We did not find marked differences in the RNA/protein ratio of the two types of gland as

reported by Baird & Herriman (1968) for rabbits. A further factor contributing to the difference may be variations in the heterogeneity of the cell population of the glands from pregnant and lactating animals.

A comparison of the protein-synthesizing activity of the polyribosome preparation from glands of lactating and pregnant animals (Table 1) showed that the latter were stimulated much more than the former by the presence of poly(U). This suggests the presence of relatively less endogenous mRNA per mg of ribosomal RNA in the preparations from pregnant animals than lactating animals, so that in the presence of a homogeneous exogenous mRNA [i.e. poly(U)] the former would be expected to be more active than the latter. Since the preparations contained a mixture of polyribosomes, monomeric ribosomes with endogenous mRNA, and monomeric ribosomes without mRNA but capable of stimulation by added poly(U), it is not possible to draw conclusions about the relative activity of the three types of ribosomes in the preparations from the two types of gland. It was, however, a consistent finding that the polyribosome preparations from pregnant animals were less active in the absence of added poly(U) than were those from lactating animals. This could have been due to the small size of the polyribosomes or there could have been an inherent defect in the ribosomes themselves, as reported by Herrington & Hawtrey (1971) for the ribosomes isolated from the glands of pregnant cows. To check this latter possibility the experiments involving ribosomal subunits and the formation of hybrid ribosomes were performed. The method used for the formation of subunits was that of Lawford (1969). In this procedure subunits are formed from run-off ribosomes, obtained by the incubation of polyribosomes with puromycin under conditions of protein synthesis. We have previously demonstrated the homogeneity of subunits

from liver and muscle prepared by this method and shown that active hybrid ribosomes could be formed (von der Decken *et al.* 1970). The present experiments show once again that not only can hybrids be formed from the ribosomes obtained from different organs of the same species of animal but also from organs of different species. This supports the conclusions of Martin & Wool (1969) who used a different method for the preparation of subunits.

The activity of recombined ribosomal subunits obtained from the glands of lactating and pregnant animals was found to be identical (see Table 2). This suggested that there was no difference between the inherent activity of the two types of ribosomes, a conclusion confirmed by the activity of the hybrids. The experiments also demonstrate the contribution made by each subunit to the activity of the ribosome and in this respect, too, there was no difference between the ribosomes from the two types of gland. This result must be interpreted with care in that the activity that is being measured is that of polymerization of phenylalanine in the presence of poly(U). It is known that this reaction does not involve the formation of the same initiation complex as does that involving endogenous mRNA (Revel, Lelong, Brawerman & Gros, 1968). Hence it is possible that the ribosomes from pregnant animals are defective with respect to the initiation of protein synthesis with endogenous mRNA but certainly not grossly defective as Herrington & Hawtrey (1971) found with bovine glands.

It is our hope that it will eventually be possible to study the conditions required to control the synthesis of α -lactalbumin by the ribosomes from mammary gland. It is therefore essential to establish that the polyribosome preparation from lactating animals is able to effect the synthesis of this specific protein. The conditions under which the polyribosomes were incubated were judged to be optimum for the synthesis of α -lactalbumin. Preliminary experiments showed that so far as the concentrations of Mg^{2+} ions, energy source and cell sap were concerned the optimum conditions for ribosomes from mammary gland were very similar to those found for liver ribosomes. Mercaptoethanol was added because it was thought possible that it might be important for the formation of the correct disulphide bonds in α -lactalbumin. Similarly the cell sap from the glands of lactating animals rather than from liver, as was used in the experiments involving ribosomal subunits, was chosen because we thought that there might be an organ-specific factor involved.

In experiments that set out to study the synthesis of a specific protein *in vitro* it is necessary to demonstrate that the synthesis is energy-dependent. Two incubations, with the same preparation of polyribosomes, were performed under identical

conditions save for the presence or absence of ATP. Although small amounts of various products containing [3H]leucine were produced during the incubation in the absence of ATP it is clear that there was no significant incorporation of [3H]leucine into α -lactalbumin (Table 3). The extensive and various chromatographic methods used for the purification of α -lactalbumin from the incubation mixture in the presence of ATP led to a fraction which was substantially radiochemically homogeneous (Fig. 4c). For the final identification of the [3H]leucine in peptides characteristic of α -lactalbumin we were fortunate to be able to utilize the fact that the protein contains only one residue of methionine and hence can be split by cyanogen bromide into two fragments. The gel-filtration chromatography for the separation of these fragments is conducted in formic acid so that the conditions are such that small radioactive contaminants would be expected to be separated. The coincidence of the radioactivity and extinction curves for the C-terminal fragment (Fig. 4d) is very satisfactory. The fact that the radioactivity per mol of leucine is much greater in the C-terminal than the N-terminal fragment is consistent with the incorporation of [3H]leucine being mainly due to chain elongation rather than the initiation of new chains. The isolation of α -lactalbumin was, of course, performed on the protein released during incubation into the soluble medium. There may be a considerable proportion of nascent α -lactalbumin associated with the ribosomes and this is currently being investigated.

According to Williams, Gurari & Rabin (1968) the enzyme discovered by De Lorenzo, Goldberger, Steers, Givol & Anfinsen (1966), which plays an important role in the formation of the correct disulphide bonds in proteins containing such links, is located in the membrane of the rough endoplasmic reticulum. In the present experiments polyribosomes freed of membrane were employed so that it is noteworthy that the native α -lactalbumin, containing at least a proportion of polypeptide chain synthesized *in vitro*, could be formed, apparently in the absence of the disulphide-interchange enzyme.

We conclude therefore from these experiments that polyribosomes from the mammary glands of lactating guinea pigs are able at least to elongate the polypeptide chain of α -lactalbumin. The way is now clear for us to examine the conditions necessary for this synthesis. Gaye & Denamur (1970) have demonstrated the synthesis of β -lactoglobulin by the isolated polyribosomes from mammary glands of lactating ewes.

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